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Cell Biology

Differential adhesion of tumor cells to capillary endothelial cells *in vitro*

(cell–cell adhesion/organ specificity/metastasis/differentiation)

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ABSTRACT **Adhesion studies were carried out to determine the relative ability of glioma cells and ovary-derived teratoma cells to adhere to endothelial cells obtained from mouse brain capillaries (designated MBE cell line) or mouse ovaries (designated MOE cell line). The teratoma cells showed preferential adhesion to MOE cells, whereas the glioma cells showed preferential adhesion to the MBE cell line. In contrast, the glioma and teratoma cells adhered equally to L929 and 3T3 fibroblasts. A testicular teratoma with ovary-seeking properties *in vivo* also adhered preferentially to MOE cells, while the preference for MBE cells was shared by glioma cells with an endothelioma and a bladder tumor line. The endothelioma, interestingly, showed a marked preferential adhesion to 3T3 cells, thus distinguishing it from the glioma. The experiments demonstrate that capillary endothelial cells derived from different sources are not alike and that differences expressed at the cell surface of these cells can be distinguished by tumor cells.**

The endothelial lining of capillaries is the first barrier to penetration by cells that are disseminated through the vascular system. Metastasizing tumor cells that have reached the bloodstream after release from their primary sites must both adhere to and pass through capillary endothelium (see refs. 1 and 2). Our working hypothesis has been that capillary endothelial cells are not all alike and that they manifest organ-associated differences reflective of their developmental history (3, 4).

We recently have obtained substantive support for this hypothesis by demonstrating through the use of both conventional and monoclonal antibodies that capillary endothelial cells express distinct organ-associated antigens on their cell surface (5). This encouraged us to examine the possibility that endothelial cells derived from different organs may be differentially and predictably recognized and adhered to by different tumor cell types. Our special interest has been to determine whether the pattern of selective adhesion, if it occurs, can be correlated with the pattern of tumor cell development and metastasis *in vivo*.

There are a variety of tests that can be used to measure selective adhesion *in vitro* (e.g., refs. 6–8). We chose for our assay a test in which tumor cells that are prelabeled with [^{3}H]thymidine are permitted to attach to endothelial cell monolayers under shear force conditions selected to favor differential adhesion (8–10). The experiments we describe demonstrate that teratocarcinoma cells and glioma cells differ in their relative adhesion to endothelial cells derived from mouse ovary and mouse brain, designated MOE and MBE cell lines, respectively, and that the preference of the teratocarcinoma cells for ovary-derived endothelium correlates with the *in vivo* seeding properties of this tumor cell line.

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MATERIALS AND METHODS

Tumors. Teratoma lines included (*i*) an ovarian teratocarcinoma of strain LT mice that had demonstrated preference for seeding to ovaries *in vivo* after intraperitoneal subcutaneous or intracardiac (left ventricle) injection (ref. 9; unpublished observations) and (*ii*) a testicular teratoma of line 129/Sv origin with demonstrated preferential seeding to ovary and spleen *in vivo* (cf. refs. 9, 11, and 12). The GL-26 glioma of C57BL/6 origin was provided by the National Institutes of Health tumor bank. An endothelioma of mouse line 129 origin was obtained from J. Hoak (University of Iowa), while the MBT-2 mouse bladder tumor of C57BL/6 derivation was provided by Y. A. Sidky (University of Wisconsin).

Cell Cultures. The mouse brain endothelial cell line MBE-1 (called MBE), isolated in our laboratory by J. Joseph (4, 5, 13), was obtained from cerebral microvessels of strain A.TL mice according to published protocols of DeBault *et al.* (14). After initial culture in modified Lewis medium (14), the line was maintained in Dulbecco's modification of Eagle's minimum essential medium (DME medium) supplemented with 15–20% fetal bovine serum and 20% S-180 tumor-conditioned medium as described by Folkman *et al.* (15). Mouse ovary endothelial cell line (MOE) was initiated by collagenase digestion of adult BALB/c mouse ovaries and maintained in tumor-conditioned medium. Endothelial cell identification included both serological and functional demonstration of angiotensin-converting enzyme (16), gross morphology of confluent culture monolayers (4, 13), ultrastructural features consistent with endothelial cell identity (5), and the ability to form tubes on prolonged cultivation *in vitro* (17).

L-929 mouse fibroblasts (from B. Kahan), 3T3(BALB/c) mouse fibroblasts (from C. Reznikoff, University of Wisconsin), and FB-356 human foreskin fibroblasts (B. Kahan) as well as GL and MBT-2 tumor cell lines were maintained in DME medium containing 10–15% fetal calf serum. FB-356 fibroblasts exposed to 5000 rad of γ-irradiation served as feeder layers for teratoma cell lines. M-5076 cells were grown in DeBault's medium (14) but could be maintained in primary culture only.

Adhesion Assays. Target cells were seeded in 24-well Falcon tissue culture plates precut into 6-well strips to facilitate later handling (7). Cultures were used for assays after the cells had been confluent for 1 day. When two different endothelial cell types were to be tested, similar passage number for each was used, and no endothelial cells were beyond passage 17. Tumor cells were prelabeled for 24 hr with [^{3}H]thymidine (10–50 μl/10 ml of medium, 1 mCi/ml; specific activity, 2.0 Ci/mmol; 1 Ci = 37 GBq). Tumor cells were passed through 20 μm Nitex filters and adjusted to a concentration of 3×10^3 cells per ml in DME medium with 15% fetal calf

Abbreviations: SI, specificity index.

serum. Tumor cells (0.5 ml) were added to test wells, and cultures were placed on a rotating platform maintained at room temperature in 5% CO_2/95% air. The diameter of rotation was equal to the diameter of the assay wells (6). Care was taken to assure simultaneous addition of different test cells to different endothelial cell monolayers and to remove wells of different assay groups from the platform simultaneously to minimize sampling errors. Immediately on removal from the shaker, the wells were gently rinsed three times with DME medium, and attached cells were then lysed by 30-min exposure to 1 M NH_4OH and assessed for radioactivity.

Data Presentation. The percent adhesion was calculated according to the formula:

$$\frac{\text{dpm sample} - \text{dpm blank}}{\text{dpm maximum} - \text{dpm blank}} \times 100$$

where 0.5 ml of the tumor cell suspension was used to obtain the maximum value, and a mean of three samples was used for all data points. Relative adhesion values for data from several experiments were obtained by using the percent adhesion of a tumor to two different target cells, with the SEM serving as a measure of interexperimental variability.

The specificity index (SI; Table 1), designed to minimize irrelevant experimental variables, was defined by the formula

$$SI = \%e_1 \frac{t_1}{t_2} \times \%e_2 \frac{t_2}{t_1},$$

where t_1 and t_2 represent two tumor types and e_1 and e_2 represent two endothelial cell monolayers. By transposition it can be seen that the SI defines equally the relative adhesion of

$$\%t_1 \frac{e_1}{e_2} \times \%t_2 \frac{e_2}{e_1}.$$

RESULTS

The key results obtained when comparing the adhesion of ovary-seeking, ovary-derived teratoma cells to that of glioma cells with respect to confluent monolayers of ovary-derived or brain-derived endothelial cells are presented in Figs. 1 and 2, and in Table 1. Fig. 1 illustrates a single experiment in which the glioma cells adhered preferentially to brain endothelium, while the teratoma cells selectively adhered to ovary-derived endothelial cells. Fig. 2 and Table 1 summarize the results of seven consecutive experiments, normalized to permit pooling of the results. The relative adhesion of the ovarian teratoma cells to ovary-derived endothelium versus that of glioma cells to brain-derived endothelium is clearly demonstrated.

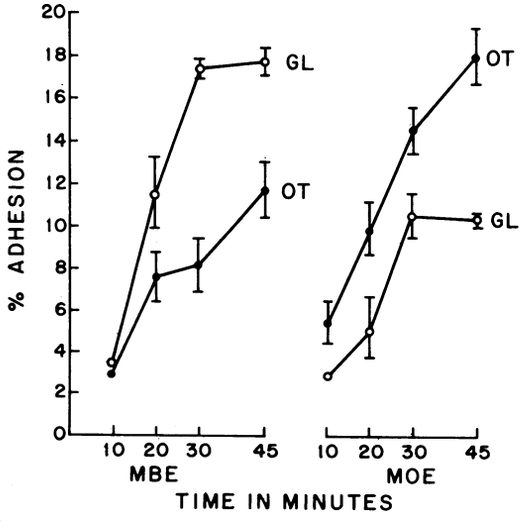

FIG. 1. Comparison of glioma (GT) and ovarian teratocarcinoma (OT) cell adhesion to MBE and MOE endothelial cells derived from mouse brain (P16) or mouse ovary (P15) microvessels, respectively (1 of 13 experiments; three samples per data point). Cultures were agitated at room temperature at a rotational speed of 100 rpm.

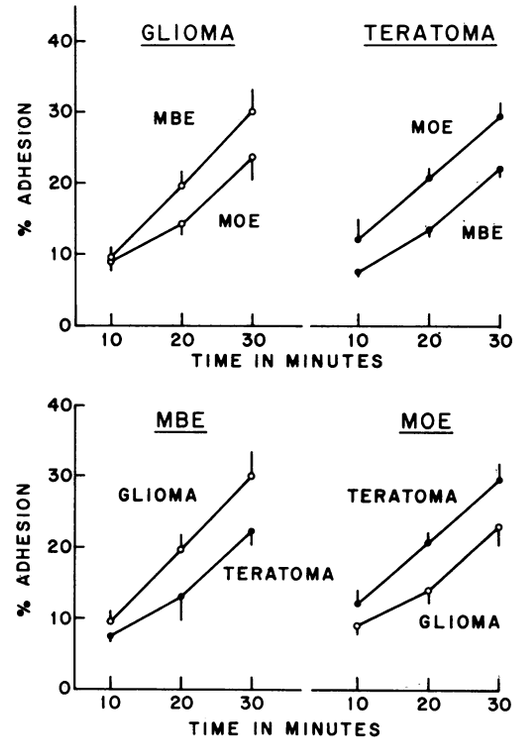

FIG. 2. Comparison of adhesion of glioma and ovarian teratocarcinoma cells to MBE and MOE cells derived from mouse brain (P8-16) or mouse ovary (P8-16) endothelium: normalized data from seven consecutive experiments (see Table 1).

Because much depended in any given assay on the conditions of the two monolayers, because the absolute number or percentage of tumor cells adhering was variable, and because one monolayer occasionally was a more efficient target cell than the other or one tumor cell type was more prone to adhesion than the other, direct comparisons became difficult. The specificity ratio was designed to reduce the impact of variables unrelated to selective adhesion by using a product of two reciprocally stated adhesion frequencies. As seen in Table 1, the SI calculations emphasize that selective adhesion was manifested in our assay system.

Comparison of the adhesion of the ovarian teratoma to MOE and MBE cells under different conditions of continuous rotation-mediated agitation is shown in Fig. 3. As the rotational speed was adjusted from 60 to 100 rpm, the absolute adhesion was reduced, but the relative adhesion preference for MOE cells was maintained. Other experiments (data not shown) indicate that a similar pattern was maintained to 125 rpm, after which the adhesion assay became variable.

Similar adhesion experiments using glioma cells indicated that a preferential attachment of MBE vs. MOE cells was demonstrable at 85–115 rpm. Below this rotational speed, the percent adherence to monolayers was too rapid and high for demonstration of specificity. At high rotational speeds,

Table 1. Relative adhesion of glioma (GL) and ovarian teratoma (OT) cells to MOE or MBE cells *in vitro*

	10 min		20 min		30 min	
	GL	OT	GL	OT	GL	OT
			% adherence*			
MBE	18.6 ± 3.5	14.6 ± 1.2	36.5 ± 2.7	24.2 ± 2.2	56.0 ± 4.6	43.0 ± 2.0
MOE	17.6 ± 3.0	22.2 ± 4.1	26.9 ± 3.1	39.8 ± 2.3	44.0 ± 4.6	57.0 ± 2.0
			Adhesion ratios			
MBE/MOE	1.06	0.66	1.36	0.61	1.27	0.76
MOE/MBE	0.95	1.52	0.74	1.64	0.79	1.32
	MBE	MOE	MBE	MOE	MBE	MOE
GL/OT	1.27	0.79	1.51	0.68	1.30	0.77
OT/GL	0.79	1.26	0.66	1.48	0.77	1.30
SI†	1.61		2.23		1.68	

Regression analysis:
GL on MBE: $y = 1.025t - 0.800$; $r = 0.99996$ GL on MOE: $y = 0.725t + 1.033$; $r = 0.98566$
OT on MBE: $y = 0.745t - 0.733$; $r = 0.98302$ OT on MOE: $y = 0.875t + 3.267$; $r = 0.99986$.

*Normalized data of percent adherence obtained from seven consecutive experiments of identical design, including three samples per data point for each experiment. Results are expressed as $\bar{X}$ ± SEM. Normalization was achieved by setting the percentage of adhesion to MOE + MBE at 30 min = 100.

†SI = GL (MBE/MOE) × OT (MOE/MBE) = MBE (GL/OT) × MOE (OT/GL).

preferential adhesion to MBE cells was not seen, although the SI calculations still indicated selectivity when data from ovarian teratocarcinoma and glioma cells were compared.

We next determined the rate of adherence of teratoma and glioma cells to fibroblast monolayers. Glioma and ovarian teratocarcinoma cells were similar in their adhesion to L929 (three experiments) and 3T3 (two experiments) fibroblasts (Fig. 4). SI calculations ranged from 0.9 to 1.1, indicating adhesion identity.

To further explore tumor cell differences in attachment to endothelial cells, we examined the relative adhesion of two teratoma cell lines, one the ovary-seeking ovarian teratocarcinoma and the other a testicular teratoma originally selected for spleen-seeking properties but subsequently found to selectively seed ovaries in female mice. As shown in Fig. 5, representing one of four experiments, the ovarian and testicular tumor cells both showed preferential adhesion to ovary-derived endothelial cells.

Comparisons with other tumors included an endothelioma (two experiments) and a bladder carcinoma (MBT-2, two experiments). Both tumors resembled the glioma GL-26 tumor in their preferential adhesion to MBE vs. MOE cells. However, the MBT-2 tumor results may have been misleading because this tumor tends towards clumping on MBE cells. Interestingly, the endothelioma showed a marked preference for adhesion to 3T3 fibroblasts (Fig. 6), a preference distinguishing this tumor from ovarian teratocarcinoma, glioma, and MBT-2 cells tested for adhesion to 3T3 cells under similar conditions of assay.

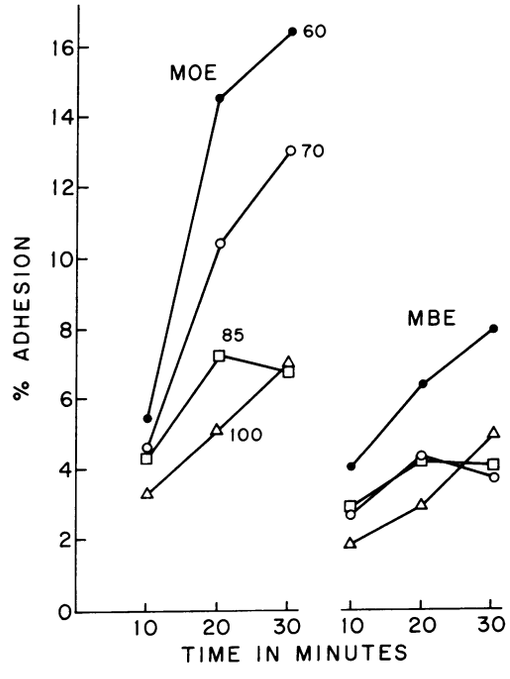

FIG. 3. Effect of rotational speed on adhesion of ovarian teratocarcinoma cells to MOE (*Left*) and MBE (*Right*) cells derived from mouse ovary (P16) and brain (P17) endothelial cells, respectively. ●, 60 rpm; ○, 70 rpm; □, 85 rpm; △, 100 rpm.

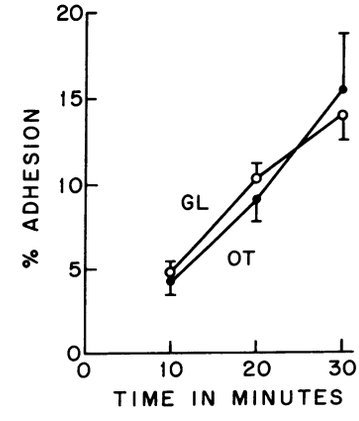

FIG. 4. Comparison of adhesion of glioma (GL) and ovarian teratocarcinoma (OT) cells to L929 (three experiments) or 3T3 (two experiments) fibroblasts. All five experiments are included. Values represent the means of % adhesion ± SEM, with three samples per data point per experiment at room temperature and 100 rpm rotational speed.

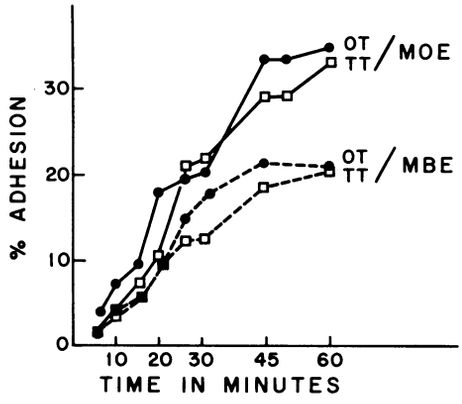

FIG. 5. Comparison of testicular (TT) and ovarian (OT) teratocarcinoma cell adhesion to MBE and MOE cells derived from mouse brain (P11) or mouse ovary (P10) endothelium, respectively (one of four experiments at room temperature and 100 rpm rotational speed).

DISCUSSION

The key finding in our study is that different tumor cells show marked differences in their adhesion to different endothelial cells. Two teratoma cell lines prefer ovary-derived endothelial cells to brain-derived ones, whereas in contrast a glioma cell line preferentially adheres to the brain-derived cells. The experiments at once confirm that capillary endothelial cells from different sources are not all alike and that differences that are expressed at the cell surface of these cells can be distinguished by tumor cells.

It is intriguing, of course, that there is a distinct correlation between the *in vivo* seeding behavior of the teratoma cells and the *in vitro* demonstrated preference. Whether, in fact, the *in vitro* system represents a valid model for the *in vivo* behavior, however, has still to be established because the total panel of endothelial cells available for study is quite limited. The preferential adhesion of the glioma to MBE cells has no direct *in vivo* parallel, although the preference for brain endothelium is consistent with the normal site of glioma growth. It is interesting that the endothelioma shows a preferential adhesion to 3T3 cells, but we have no ready suggestion for why this should be the case. Clearly, more metastatic variants are needed along with a broader representation of capillary endothelial cells. Moreover, the need to establish endothelial cells from lymphatic vessels, likely to be involved in metastatic spread of carcinoma cells, is a pressing one.

There are considerable technical limitations in our adhesion assays. Tumor cells are not necessarily identical from day to day although their long-term stability in culture and our rigid transfer protocol tended to minimize variation. Some tumor cells required feeder layers; one did not survive long-term culture, and media requirements varied. More importantly, the behaviors of different endothelial cell lines are not identical: MBE cells grow more rapidly than MOE cells, confluent monolayers are not always identical, and timing to have all cells at the right stage of culture at one time is difficult. Under the circumstances, the consistency of consecutive experimental runs, listed in Table 1, shown both by the small range of SEMs and by the consistently high correlation coefficients in regression analysis, is encouraging.

Three-way analysis of variance, carried out on selected experimental runs, demonstrated that speed of rotation, time of assay, and type of tumor and endothelium combination all influenced the percent adhesion values. The choice of 100 rpm, falling within a comparable range of 85–115 rpm, appears justified. At lower speeds, nonspecific adhesion and a high rate of total adhesion seem likely to be less representative of the *in vivo* situation, where only a small fraction of the total tumor cell population is expected to seed to a given organ (18), whereas high speeds of rotation lead to an uneven distribution of tumor cells and an instability of cell adhesions.

The selective mechanisms responsible for preferential metastasis of tumors to various organs in the body have not been well defined (cf. refs. 1, 2, 19, 20). Fidler, Hart, Poste, and their colleagues have developed melanoma lines that differ in their metastatic preferences (1, 20–23). Our own studies and those of Kahan have focused on sublines of teratomas (9, 11, 12). In both systems, monolayer adhesion assays have given some indication of specificity: Kahan's studies used whole organ explants, however (9, 11), thus preventing identification of specific cell types responsible for the limited specificity seen, whereas Nicolson and his colleagues (10, 24–26) focused attention on the subendothelial matrix. Recently, however, Nicolson (cited in ref. 26) has begun to examine the specific role played by endothelial cells in mediating tumor cell adhesion.

The clearest example of endothelial cell specificity in relation to extravasation of circulating cells is seen in the lymphocyte homing studies of Woodruff and her colleagues,

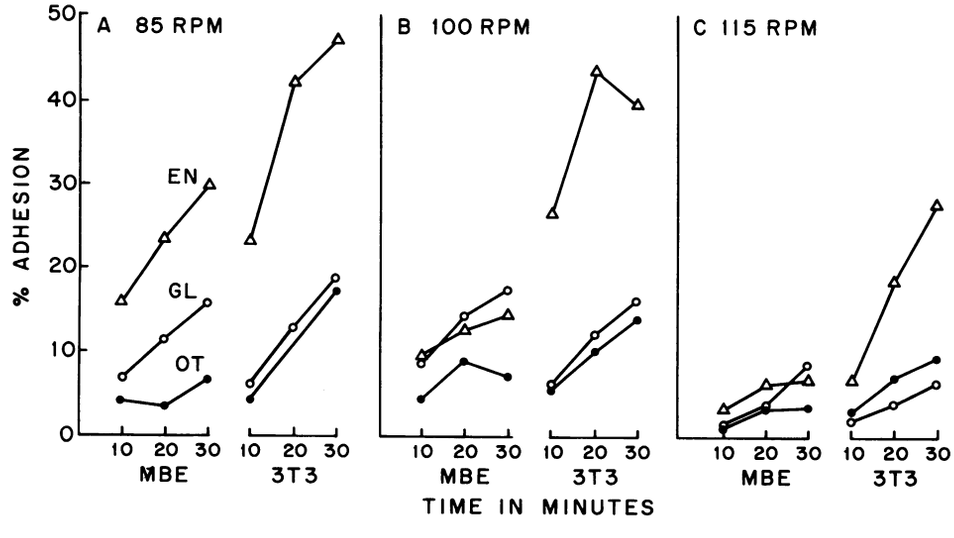

FIG. 6. Comparison of adhesion of endothelioma (EN), glioma (GL), and ovarian teratocarcinoma (OT) cells on MBE (P11) cells and 3T3 fibroblasts.

who have shown convincingly that vascular cells of the thoracic duct, spleen, and lymph nodes but not of thymus have specific high endothelial cell adherence molecules on their surface, blockable by specific inhibitors and critical for effecting lymphocyte adherence to and penetration through the specialized endothelial cells (27–29). Butcher, Scollay, and Weissman, extending these observations, have demonstrated that there is organ specificity in this lymphocyte–endothelial cell interactive system, for subsets of lymphocytes preferentially adhere either to Peyer's patch or lymph node endothelium (30–33). In their studies, moreover, thymic lymphomas have been used whose organ selectivity *in vivo* is paralleled by their selective adhesion in the experimental situation. Most recently, their general conclusions have been supported by studies using monoclonal antibodies generated against cell surface antigens expressed on lymph node endothelial cells (34).

In our own studies, we have seen antigenic specificity on endothelial cell surfaces that correlate with the organ in which these are found (5). Earlier studies of Pressman and his colleagues using conventional antibodies (35) as well as more recent work with various lectin-binding sites (36) document cell surface-associated differences among vascular endothelial cells.

We believe it likely that the most relevant information for understanding the selective adhesion of tumor cells to endothelium will come from analysis of embryonic systems (37–41), for it is in these that much progress has been made in characterization of the factors responsible for cell adhesion. The parallels between tumor cell development and embryogenesis have been discussed frequently (e.g., refs. 42 and 43), but they take on special significance as we begin to identify the specific cell surface molecules that mediate adhesion (44–49). While there are certainly many other factors that influence tumor cell–host interactions (cf. refs. 1, 2, 48, 50–52), tumor cell adhesion to the vascular endothelium must represent a critical early step leading to the establishment of metastases.

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